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Isolation and Phenotypic Characterization of Phosphate Solubilizing Bacteria from Lentil (*Lens culnaris*.) Rhizosphere Soils from Southern Parts of Tigray, Ethiopia

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Abstract: The use of Phosphate solubilizing microorganisms as a phosphorus biofertilizer improves the soil fertility and increases the crop production, which is mostly achieved by Rhizobium phosphate solubilizing bacteria. The objective of this study was to isolate and phenotypically characterize the beneficial Rhizobium bacteria from root nodules of *lentil rhizospheres*. A total of 16 soil samples were collected from two districts of South Eastern Tigray Zone, northern Ethiopia. Nodules were collected from lentil roots after 45 days of growth in the green house. A total of eight rhizobia bacterial strains were isolated and identified by employing standard morphological, cultural, biochemical and physiological characterization techniques of identification for phosphate solubilizing bacteria. All strains were gram negative, motile and did not absorb Congo red. On the other hand, the tested isolates were negative for citrate, urease and positive for hydrogen sulphide tests. Similarly, all the isolates had negative response for methyl red test except EmPSB-2 which was positive. Half of the isolates (EmPSB-1, HhPSB-6, HhPSB-7 and HaPSB-8) showed positive result to TSI while the remaining showed negative. All of the isolates showed positive result to catalase test. The rhizobial isolates were able to grow at YEMA supplemented with 2% NaCl salt concentrations and the most salt tolerant isolate was (EmPSB-2) which was able to grow up to 8% NaCl and the least salt tolerant strains were EkPSB-3 and HaPSB-8. However, all the isolates did not grow at maximum salt concentration. Furthermore, all of the isolates were unable to grow at pH =4 and 10 except for the isolates EmPSB-1 and HaPSB-8 which showed low growth performance at pH=10. However, good growth rate were recorded at pH=7 for all strains of bacteria. The EkPSB-3 isolate showed maximum solubilizing index (3.5mm) when grown in YEMA culture media supplemented with tricalcium Phosphate and the isolate HhPSB-6 showed least phosphate solubilization index (2.4mm). In general, based on the obtained data, eight isolates (EmPSB-1, EmPSb-2, EkPSB-3, EmPSB-4, HdPSB-5, HhPSB-6, HhPSB-7 and HaPSB-8) were identified and phenotypically characterized. This study recommend further test at green house level for its effectiveness on phosphate solubilization or as bioinoculants by testing them on different crops.

Key words: Lentil Roots · Psb · Rhizobia and Yema

INTRODUCTION

Lentil belongs to the genus Lens of the Viceae tribe in the Legumnosae (Fabaceae) family, commonly known as the legume family [1]. Currently, lentil becomes an important pulse crop throughout the world [2, 3]. Ethiopia is amongst the centers of diversity for lentil and one of the major lentils producing countries in the world and the first in Africa [4]. Nutritionally, it is an invaluable source of protein for the vast majority of Ethiopian people [5]. Therefore, improving the production of this crop using different methods will contribute to the food security of the country. Phosphorus (P) is a major growth-limiting nutrient; which is the least available and least mobile mineral nutrients for plants in the soil [6]. The deficiency is also the main problem of Ethiopian soils particularly highland soils. Around 70% of Ethiopian vertisols have available phosphorus below 5 ppm, which is very low for supporting good plant growth and fixation in vertisols is related more to calcium, which is the predominant cation in all profiles than Al^{3+} and Fe^{3+} [7]. Previous researches also indicated that phosphate solubilizing microbes

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(Especially bacteria) are very important in response to phosphorus deficiency in the soil. The isolates can be used to solubilize an insoluble phosphate, which is characteristic of Ethiopian highland soils. These PSM (Phosphate solubilizing microbes) are found in soil most likely in legume roots in which nodulation takes place. Though chemical fertilizers have played their role in modern agriculture, the current escalating price and use below the recommended amount are the main limiting factors for most Ethiopian farmers for better production of crops [8]. So that, there is an urgent need to use alternatives and cheaper sources of nutrients particularly those of nitrogen and phosphorus that limits crop production in Ethiopia and the cause for low yield [8]. One of the cheap alternatives for improving the deficiency of phosphate nutrition is the manipulation of phosphate solubilizing microbes (PSM) alone or together with cheap rock phosphate [9]. This is done by isolating the competitive and the best PSB strains from legume roots (Such as lentil) to develop and manufacture biofertilizer [10] and transfer the technology for the farmers. Hence, this study was initiated with the aim of isolating and characterizing efficient phosphate solubilizing bacteria from lentil root nodules.

MATERIALS AND METHODS

Study Sites and Soil Samples Collection: Preliminary survey and soil sample collection were done in Enderta and Hintallo Wajrat districts of Southern Eastern Tigray, Ethiopia based on the potential of the place for lentil growth. From each districts a total of 16 sub districts ("Tibias") were selected for soil sample collection by considering their location for transport access. The soil samples were excavated following cross sectional study from a depth of 0 -30 cm from lentil grown farms and separately collected in sterile polyethylene plastic bags. Each soil sample was grounded into smaller parts and sieved using 2 mm pore size sieve and 3kg of 8 composite soil samples were dispersed in alcohol sterilized pots and put in triplicates. Lentil seeds were collected from Mekelle Agricultural Research Center and surface sterilized with 95% ethanol and 0.1% $HgCl_2$ solution for three minutes and washed five times with sterilized distilled water according to Vincent [11]. Surface sterilized seeds were allowed to germinate on 5ml of sterile water for three days at room temperature. Five peregrinated seeds were selected and planted on the prepared pots, which were thinned down into three seedlings after five days of emergence. All pots were situated at Mekelle University green house over the table and watered to a field capacity every three days interval for 45 days.

Isolation of Phosphate Solubilizing Bacteria: After 47 days of emergence, all the plants were carefully uprooted from the pots and large and brown root nodules were separately collected from lentil roots. After surface sterilization the nodules were transferred in to sterile petridishes containing 5ml of sterilized distilled water and crushed using sterilized glass rod. A milky suspension of bacteria's were collected from the nodules and preserved in to 24 different test tubes under 0.3% of $CaCO_3$ solution for each representative districts and sub districts and stored in a refrigerator at 4°C. A loopful of the extracts was streaked on YEMA (Yeast Extract Manitol Agar) media and incubated at 28±2°C for 3-5 days. From each plate, single typical rhizobia colony were picked and purified by re-streaking in to new YEMA plates. Pure isolates were then preserved on YEMA slants containing 0.3% of $CaCO_3$ stored at 4°C for short term storage [11] by designating the test tubes using name of collection area.

Characterization of Isolates: All the identified bacterial isolates were tested for biochemical (Catalase test, Triple Sugar Iron (TSI) test, Urease utilization test, Citrate Utilization test, Methyl red test, H_2S production test), physiological (pH and salt tolerance) and morphological (Gram staining, motility, colony color, colony arrangement, mucus production and shape), according to Benson [12].

Congo Red Absorption Test: The isolates were tested on YEMA agar containing 1% of Congo red and incubated from 3 to 5 days at 28°C. The colonies were characterized based on the absorbance of the red coloration [13].

Phosphate Solubilizing Ability Test: This was determined by inoculating the isolates on YEMA supplemented with tricalcium phosphate. This was checked based on growth and the presence of clear zone (Halo zone) formation around the colonies [14]. Growth and clear zone qualitatively recorded as (+) for growth and clear zone (-) for no growth and quantitatively analyzed by calculating its solubilization index.

$$SI = (HD + CD)/ CD$$

Where; SI= Solubilization index, HD= Halo zone diameter, CD= Colony diameter

Data Analysis: All experiments were set in triplicates and the data was an average of the three. Symbiotic data were analyzed using descriptive statistics.

RESULTS AND DISCUSSION

Isolation of Phosphate Solubilizing Bacteria from Different Locations: A total of eight phosphate solubilizing rhizobia bacteria were obtained from 16 composite soil samples collected from lentil growing areas of South Eastern Zone of Tigray.

Cultural and Growth Characteristics of Isolates: As indicated in Table 1, growth, morphological and cultural characteristics of all the isolates were done. Accordingly, all the rhizobia isolates were gram negative having bacilli colony arrangement. Our findings are in line with many reports [15-18]. With regard to colony morphology, isolates EmPSB1, HhPSB-7 and HaPSB-8 were smooth whereas, EkPSB-3, EmPSB-4 and HdPSB-5 were morphologically creamy on YEMA medium. On the other hand, EmPSB-2 and HhPSB-6 isolates were rough. The growth of the isolates EmPSB-1, EkPSB-3, EmPSB-4 and HdPSB-5 were large mucoid and the remaining isolates were medium mucoid on YEMA medium. The shapes of all the isolates were rod where EmPSB-1, EmPSB-4 and HhPSB-6 were long rod, whereas EkPSB-3, HdPSB-5 and HhPSB-7 were short rod; EmPSB-2 and HaPSB-8 was normal rod. Similar morphological and cultural characterization of rhizobia bacteria were discussed by Mulugeta Fentahun *et al*. [17] on rhizobia isolated from haricot bean root nodules.

Moreover, the isolates EmPSB-1, EkPSB-3, EmPSB-4 and HdPSB-5 had whitish colony color; likewise, EmPSB-2, HhPSB-6, HhPSB-7 and HaPSB-8 showed Yellowish color. The difference in cultural and growth characteristics may be due to the genetic variation among the isolates [19, 20].

Biochemical Test for the PSB Isolates: The results shown in Table 2 indicated that all rhizobia isolates were motile. Whereas the isolates grown on YEMA supplemented with Congo red failed to absorb Congo red and stand out as white and translucent. Similar results were obtained by Bhatt *et al*. [15] and Mulugeta Fentahun *et al*. [17]. On the other hand, except EmPSB-2, all the isolates did not produce acid when tested on Methyl red test. The results were in agreement with the earlier finding obtained by Bhatt *et al*. [15]. Out of the eight isolates tested on urease, citrate, hydrogen sulphide and methyl red test were negative except the isolate EmPSB-2 which was methyl positive. Similar findings were obtained by Bhatt *et al*. [15] and Shahzad *et al*. [21]. In triple iron sugar test, half of rhizobial isolates (EmPSB-2, EkPSB-3, EmPSB-

Table 1: Growth, morphological and cultural characteristics of the *Rhizobium* isolates

No	Isolates	Growth on YEMA	Colony morphology	Colony color	Colony arrangement	Gram stain test	Colony shape
1	EmPSB1	Large mucoid	Smooth	Whitish	Bacilli	Gram -ve	Long rod
2	EmPSB2	Medium mucoid	Rough	Yellowish	Bacilli	Gram -ve	Rod
3	EkPSB3	Large mucoid	Creamy	Whitish	Diplobacilli	Gram -ve	Short rod
4	EmPSB4	Large mucoid	Creamy	Whitish	Bacilli	Gram -ve	Long rod
5	HdPSB5	Large mucoid	Creamy	Whitish	Bacilli	Gram -ve	Short rod
6	HhPSB6	Medium mucoid	Rough	Yellowish	Bacilli	Gram -ve	Long rod
7	HhPSB7	Medium mucoid	Smooth	Yellowish	Bacilli	Gram -ve	Short rod
8	HaPSB8	Medium mucoid	Smooth	Yellowish	Bacilli	Gram -ve	Rod

NB. PSB= Phosphate Solubilizing Bacteria, Em(1)= Enderta mykeyah, Em(2)= Enderta mytsedo, Ek= Enderta kedamaywoyane, Em(4)= Enderta Mysenti, Hd= Hintallo wajirat dejen, Hh(6)= Hintallo wajirat hintalo, Hh(7)= Hintallo wajirat hewane and Ha= Hintallo wajirat amdewoyane

Table 2: Biochemical characteristics of phosphate solubilizing bacterial (PSB) isolates from rhizosphere soil of lentil from South Easter Tigray

	PSB Isolates							
Tests	EmPSB-1	EmPSB-2	EkPSB-3	EmPSB-4	HdPSB-5	HhPSB-6	HhPSB-7	HaPSB-8
TSI	+	-	-	-	-	+	+	+
Motility	+	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	-	-	-	-
H_2S production	+	+	+	+	+	+	+	+
Methyl red	-	+	-	-	-	-	-	-
Congo red	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+

NB. TSI= Triple sugar iron

Table 3: Growth of PSB isolates on different pH range

		pH						
No	Isolates	4	5	6	7	8	9	10
1	EmPSB-1	-	±	+	++	+	+	±
2	EmPSB-2	-	+	±	++	+	+	-
3	EmPSB-3	-	-	+	++	+	±	-
4	EmPSB-4	-	±	±	++	++	-	-
5	HdPSB-5	-	-	+	++	+	-	-
6	HdPSB-6	-	±	±	++	+	±	-
7	HdPSB-7	-	-	+	++	+	-	-
8	HdPSB-8	-	+	+	++	+	+	±

N.B:- *(-; no growth; ±: Low growth; +: average growth; ++: good growth)*

4 and HdPB-5) were fermented glucose produce gas in the test tubes and the remaining four isolates were fermented lactose and glucose. Esubalew Sinte [22] reported that 95% of phosphate solubilizing bacteria grew in the presence of glucose. Likewise, Nautiyal [23] argued that the most efficient strain is the one that is capable of utilizing a wide range of carbon and nitrogen sources. On the other hand, all the isolates showed positive for catalase production test. In agreement with the present findings, different reports identified that all the tested rhizobia isolates were capable of producing the enzyme catalase [18, 21].

Physiological Test: Each rhizobia isolates were physiologically tested on different salt concentration, pH range and phosphate solubilizing ability. The lentil nodulating rhizobia tested showed disparity response in different pH medium (pH 4-10). All tested isolates had good growth performance at pH range 7-8. Except the isolates EmPSB-1 and HaPSB-8, which showed low growth performance at pH=10, none of the isolates were able to grow at pH=4 and 10. In general, the isolates EmPSB-1, EmPSB-2, EkPSB-3, HdPSB-5, HhPSB-6 and HaPSB-8 showed average growth performance at pH range of 5-9. Among all the tested isolates good growth performance were recorded on isolate HaPSB-8 which can be recommended as an inoculants at both acidic and alkaline soils. The results were in agreement with the earlier finding obtained by Mulugeta Fentahun *et al*. [17] on isolation and characterization of nitrogen deficient rhizobium on haricot bean. It was also shown by Ayalew [24] that the soil acidity was the major limiting factor for the production of leguminous crops in Ethiopia. Therefore, these acid tolerant *Rhizobium* isolates may play a great role in improving the yield and production of leguminous crops in this region. The growth of the isolates on a wide range of moderate acidity and alkalinity (pH 5 to 10) is concurrent with the findings that showed R. *leguminosarum* bv.*viciae* strains are generally sensitive to low pH and grow well on near neutral and basic pH [9, 25, 26]. The variation in growth at different pH concentration might be a relation between pH of origin of isolates and their acidic and alkaline pH tolerance from which most isolates recovered. Similar works have been also done by Harun *et al*. [27] who found that there is a great variation among lentil nodulating rhizobia with respect to growth and survival in acidic and alkaline conditions. They showed that they can grow well at acidic pH as low as pH 4 and alkaline pH as high as pH 10.

All the isolates were grown on YEMA supplemented with 1 to 2% of NaCl salt concentration (Table 4). This finding is in line with the work of Mulugeta Fentahun *et al.* [17]. The most salt tolerant isolate was EmPSB-2, which was able to grow at salt concentrations of 1 up to 8% [28] reported that fast growing rhizobial isolates could tolerate more than 2% of NaCl concentration than slow growing isolates. However, in this study the most salt sensitive isolates were EkPSB-3, HdPSB-5 and HaPSB-8 which were able to grow at only 1 and 2% of NaCl. This finding is similar with the findings of Esubalew Sinte [22] who reported that phosphate solubilizing nodule bacteria from lupins were able to grow at 2%. On the other hand, the remaining isolates (EmPSB-1, EmPSB-4 and HhPSB-6) showed moderate performance which grows between 1-4 % and 1-6%, respectively.

Phosphate Solubilizing Ability: All of the eight isolates showed halozone around the colonies when grown in YEMA culture media supplemented with Tricalcium phosphate (Table 5). The colony diameter, halo zone diameter and solubilization index (SI) upon 2- 8 days of incubation were measured. In the present study, the maximum halozone (7mm each) were formed on the isolates EmPSB-1, EmPSB-2 and HhPSB-6. However, the minimum halozone (4 mm) was measured on the isolate HaPSB-8 within eight days of incubation. In general the

Table 4: Effect of *Rhizobium* isolates on the salt tolerance (NaCl)

No	Isolates	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%
1	EmPSB-1	+		+	+	-	-	-	-	-	-
2	EmPSB-2	+		+	+	+	+	+	+	-	-
3	EkPSB-3	+	+	-	-	-	-	-	-	-	-
4	EmPSB-4	+	+	+	+	-	-	-	-	-	-
5	HdPSB-5	+	+	-	-	-	-	-	-	-	-
6	HhPSB-6	+	+	+	+	+	-	-	-	-	-
7	HhPSB-7	+	+	+	+	+	+	-	-	-	-
8	HaPSB-8	+	+	-	-	-	-	-	-	-	-

N.B '-'No growth and '+' abundant growth

Table 5: Measurement of Halo and Colony Diameters of Isolates upon 8 Days incubation

	Day 2 Diameter(mm)			Day 4 Diameter(mm)			Day 6 Diameter(mm)			Day 8 Diameter(mm)		
Isolates	CD	HD	SI	C	H	SI	C	H	SI	C	H	SI
EmPSB-1				2	3	2.5	3	6	3	4	7	2.8
EmPSB-2				2	4	3	3	5	2.7	3	7	3.3
EkPSB-3				1.5	3	3	2	5	3.5	2	5	3.5
EmPSB-4	1	1	2	2	2	2	3	4	2.3	4	6	2.5
HdPSB-5				1	1	2	2	3	2.5	3	6	3
HhPSB-6				2	3	2.5	3	4	2.3	5	7	2.4
HhPSB-7	2	2	2	1	1	2	2	5	3.5	3	5	2.7
HaPSB-8				1	2	3	1	2	3	2	4	3
Average				1.5	2.3	2.5	2.3		2.7	3.3	5.8	2.9

average solubilizing index of the isolate was 2.9 mm. This result is in agreement with the report of Yasmin and Bano [18].This is due to the production of organic acid in the surrounding medium [29].

As described by different researchers, the formation of the clear zones and measurement of SI on a solid medium is a very important and reliable tool for a preliminary screening of a large number of phosphate solubilizing micro-organisms [20, 30]. According to [31] isolates with high solubilization index are good phosphate solubilizers while [19] reported that the isolates showing higher and lower solubilization index on a solid medium are good phosphate solubilizers. With little or no halo zone formation around the colonies tested on liquid medium supplemented with tricalcium phosphate source, Nautiyal [23] and [32] found that bacterial strains with highest efficiency of phosphorus solubilization in the qualitative (Plate) assay method.

CONCLUSION

Rhizobium, the phosphate solubilizing bacteria are efficient to improve soil nutrient, property of soil and environment. The isolates identified from the two districts (Enderta and Hintalo Wajirat) showed different phenotypic characters tested for morphological, biochemical and physiological test. Based on morphological, biochemical and physiological characteristics, eight isolates were obtained with different solubilizing efficiency. From these isolates some of them also showed significant difference potential to grow under salinity and pH stress conditions which might be candidates for their utilization both under extreme pH condition as well as in saline soils.

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